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FLOW CYTOMETRIC DETERMINATION OF THE PHOTOINDUCED TOXICITY OF ANTHRACENE TO THE GREEN ALGA *SELENASTRUM CAPRICORNUTUM*

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Abstract—Certain PAHs are photosensitizers and in the presence of solar radiation can cause toxicity to aquatic plants and animals. The photoinduced toxicity of anthracene to the green alga *Selenastrum capricornutum* was assessed by the use of flow cytometry to measure cell size, cellular chlorophyll concentration, and cell viability. Anthracene was slightly toxic in the absence of UV-A radiation. The detection of the direct toxicity of anthracene in this study at a concentration of 19 μg/L anthracene resulted from the use of sensitive flow cytometric measures. There was a significant interaction between anthracene and UV-A radiation, which, in combination, caused significant toxic effects on *Selenastrum capricornutum*. The most sensitive flow cytometric measure of toxicity was the stress index (SI), which was predictive of longer term effects on cell growth. The 28-h EC50 and EC10 for the SI for *Selenastrum capricornutum* were 16.1 and 8.3 μg/L anthracene, respectively, at 125 $\mu W/cm^2$ UV-A. All combinations of anthracene and UV-A that inhibited algal growth also caused a significantly greater number of nonviable cells. The flow cytometric methods used in this study proved to be sensitive, predictive measures of the direct and photoinduced toxicity of anthracene and UV-A radiation to *Selenastrum capricornutum*.

Keywords—Anthracene PAH Ultraviolet radiation *Selenastrum* Flow cytometry

INTRODUCTION

PAHs are ubiquitous pollutants that are produced as a result of natural and human processes. PAHs in aquatic environments are associated with point and nonpoint discharges such as fossil fuel combustion, automobile emissions, steel and coking operations, and petroleum refining [1].

Photoinduced toxicity, in which the toxicity of PAHs is enhanced by simultaneous exposure to UV radiation, has been recognized by the biomedical community for many decades [2,3]. However, it was not until recently that the photoinduced toxicity of PAHs was demonstrated with standard aquatic bioassay organisms [4–7]. The photoinduced toxicity of PAHs has been observed in a variety of aquatic organisms at PAH concentrations well below their aqueous solubility [6–9]. Specifically, photodynamic effects of PAHs have now been demonstrated in fishes [5–8], daphnids [7,9], algae [10–12], and aquatic angiosperms [13]. The photoinduced toxicity of anthracene, a linear three-ring PAH, has been characterized for fishes [5,14,15], aquatic insects [6,7], and zooplankton [4,6,7,16,17]. Other PAHs that have exhibited photoinduced toxicity due to simultaneous UV radiation exposure include fluoranthene, pyrene, benzo[*a*]pyrene, benz[*a*]anthracene, acridine, and benzo[*a*]fluorene [7–9,16]. The environmental hazard of PAHs has been underestimated because all of these studies have observed enhanced toxicity of PAHs with simultaneous exposure to environmentally relevant wavelengths and intensities of UV radiation. Hazard assessments of the photoinduced toxicity of PAHs [11,14,17] suggest that aquatic systems heavily contaminated with PAHs may be experiencing increased mortality and reduced reproductive success of aquatic organisms.

Previous studies of the photosensitizing effects of PAHs on algae have indicated that algae are more tolerant of these effects than other aquatic organisms [6,10]. The inability of previous studies to demonstrate significant phototoxic effects of PAHs on algae is probably due to relatively insensitive end points [6] and inappropriate exposure conditions [10]. Gala and Giesy [11] were the first to demonstrate that algae are sensitive to photoinduced toxicity caused by anthracene at ecologically relevant intensities of UV-A radiation and anthracene concentrations less than water solubility (35 μg/L). Both population growth rate and primary production were sensitive to and inhibited by the photoinduced toxicity of anthracene [11]. However, *Selenastrum capricornutum* seemed to be fairly tolerant of effects on cellular photosynthesis ($[^{14}C]$bicarbonate incorporation on a per-cell basis) [11]. Until the research reported here, growth rate was the most sensitive response to the photoinduced toxicity of anthracene that had been measured in algal cells.

The importance of algae in aquatic food chains requires that an accurate and sensitive characterization of the chronic photoinduced toxicity of PAHs to algae be determined such that phytoplankton can be included in hazard assessments of the photoinduced toxicity of PAHs to aquatic ecosystems. It has already been established that solar UV radiation (290–400 nm) can inhibit primary production and damage algal

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cells [18,19]. Therefore, we used sensitive flow cytometric measurements to assess the chronic effects of UV-A radiation and phototoxic effects of anthracene on *S. capricornutum*. The responses of algae to toxicants are typically studied using population-based parameters such as specific growth rate, biomass, cell yield, and primary production [11,18]. Flow cytometry offers a sensitive alternative to these standard measurements because it allows the rapid and quantitative measurement of responses of individual cells [20,21]. Flow cytometry can make measurements on algal cell size, shape, chlorophyll content, and physiological condition of cells [21–24].

Here we present the results of experiments designed to determine the effects of anthracene in the presence of UV-A radiation on several flow cytometric end points in the green alga *S. capricornutum*. We evaluated the relative sensitivities of the flow cytometric measures and compared them to more standard measures of the responses of algal cells to toxicants. Furthermore, we used the flow cytometric measures to investigate the mode of toxic action of anthracene in the presence of UV-A radiation to *S. capricornutum*.

MATERIALS AND METHODS

Test organism

The test alga was the unicellular freshwater green alga, *S. capricornutum* Printz, which is the standard freshwater alga used in bioassays of the effects of both nutrients and toxicants [25]. The algal culture and test medium, U.S. Environmental Protection Agency (EPA) algal assay medium [25], was used double strength (2×) to increase the bicarbonate buffering capacity of the medium and to prevent algal cultures from becoming carbon or nutrient limited during the toxicant exposure. Algal cultures and all test exposures were maintained at 21 ± 1°C.

Lighting

The lighting consisted of continuous photosynthetically active radiation (PAR) from above and a UV-A illumination from below [11]. The PAR source was six Chroma F40C50 (General Electric) white fluorescent bulbs covered by a sheet of Plexiglas® UF-3 (Rohm and Haas, Philadelphia, PA), which eliminated all wavelengths <390 nm. UV-A radiation was supplied by four F40 BLB (General Electric) black-light fluorescent bulbs. A sheet of 0.005-mm-thick Mylar® (DuPont) was used to eliminate wavelengths shorter than 315 nm because UV-B radiation (280–320 nm) is toxic to photosynthetic organisms [18,19]. The F40 BLB bulbs were selected for the UV-A source because the spectral characteristics of the F40 BLB bulbs (Fig. 1) match the action spectrum for the photoinduced toxicity of anthracene, which is between 320 and 380 nm with peak activity around 360 nm [4,26]. The test vessels were Pyrex® Erlenmeyer flasks because Pyrex glass is transparent to wavelengths >310 nm, as measured by a Gilford (Oberlin, OH) 2600 UV/vis spectrophotometer (referenced to air). Neutral density screening was used to reduce UV-A radiation to the desired intensities, which ranged from 125 to 765 $\mu W/m^2$ UV-A. UV-A radiation was quantified using the UV-A (365 ± 36 nm) probe of the Macam Photometrics (Livingstone, Scotland) model UV-103 radiometer. PAR (400–700 nm) was quantified using the Li-Cor (Lincoln, NE) LI-192s quantum sensor coupled to an LI-150 integrator.

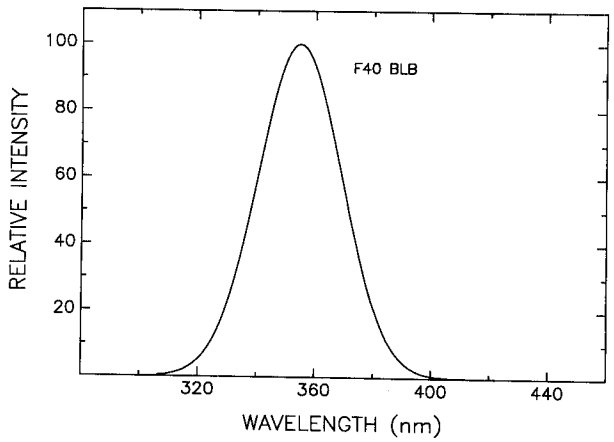

Fig. 1. Spectral characteristics of General Electric F40 BLB black-light, fluorescent lamps (modified from General Electric).

Toxicant

Stock solutions of anthracene (Sigma [St. Louis, MO] grade III, lot 84F-34, 99.9% purity) were prepared in HPLC-grade acetonitrile. A carrier solvent was necessary because a toxicant renewal system was chosen to maintain constant anthracene exposure concentrations. Acetonitrile was chosen as the carrier solvent because it is completely miscible with water, is photochemically inert, does not transfer triplet energy, and does not absorb UV radiation present in the solar spectrum or in the artificial lighting used in this study [27].

The experimental design and test conditions were identical for all exposures, except that the UV-A intensity and concentration of anthracene were systematically varied among experiments. Each experiment consisted of four treatments: an acetonitrile control treatment and three anthracene exposure concentrations. Each treatment consisted of three replicate flasks.

One hundred milliliters of filter-sterilized (0.45-μm membrane filter) 2× algal medium was added to each chemically cleaned, autoclaved 250-ml Pyrex Erlenmeyer flask. Concentrations of anthracene were maintained by using a toxicant-renewal exposure system in which small volumes of anthracene stock solutions were periodically added to each flask to maintain the nominal concentration, because there was rapid and continuous photolysis of anthracene (Fig. 2). This obviated the need for a continuous-flow system, which could have washed cells out of the system. Toxicant renewal occurred at the end of the preincubation period and every 8 h during UV-A exposure. Anthracene stock solution was added to bring the three replicate flasks for each anthracene treatment to their target anthracene concentration. The target anthracene concentrations for the three treatments were 35, 15, and 7 $\mu g/L$. The control treatment received a quantity of acetonitrile equal to the maximum volume added to the three anthracene treatments during each toxicant renewal

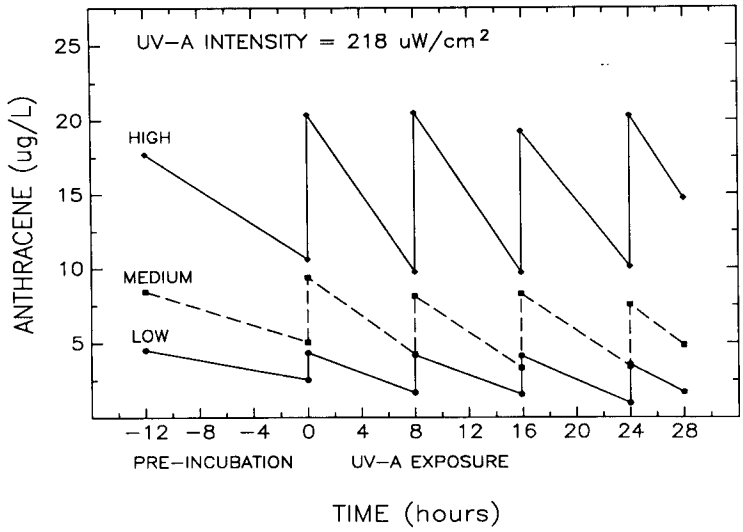

Fig. 2. Anthracene concentration in test media during an experiment conducted at 218 $\mu W/cm^2$. The target concentrations for the three treatments were 35, 15, and 7 $\mu g/L$.

period. During the exposure period, all four treatments were exposed to UV-A radiation.

Before each renewal, the mean anthracene concentration for each treatment was determined. The volume of anthracene stock solution required to elevate the anthracene concentration back to the target anthracene concentration was added to the treatment flasks. The maximum volume of acetonitrile added during any renewal never exceeded 50 μl per flask. The anthracene loss rate and concentration required to reestablish the nominal concentrations were calculated assuming anthracene loss followed first-order kinetics between renewals [17]. The predicted concentrations (0.1-h intervals) were summed and averaged for the desired exposure duration (Fig. 2).

Anthracene concentrations in media were measured at the beginning of the preincubation and before and after each toxicant renewal. Anthracene concentrations in the test medium were determined directly by reversed-phase HPLC after centrifugation for 10 min at 2,000 *g* to remove algal cells [11]. The detection limit of anthracene in water was 0.1 $\mu g/L$. Anthracene concentrations in the control treatment flasks were always below the detection limit.

Standard algal toxicity test conditions were modified slightly for these experiments. One milliliter algal inoculum was added to each flask such that the starting algal density was 1×10^5 cells per milliliter. The flasks were placed under the PAR lighting (47–53 $\mu M/m^2/s$), in the absence of UV-A radiation, for the 12-h preincubation period. The preincubation period enabled the algal cells to reach steady state with the anthracene in the algal medium before UV-A exposure [28,29].

Flow cytometry

The principle of operation of a flow cytometer is the hydrodynamic focusing of cells in solution into a fluid stream so narrow that the cells are carried along in single file through a laser beam (Fig. 3) [24,30]. The spot size of the laser beam is small enough that only one particle (algal cell) is illuminated at a time. With the proper configuration, filters, and photomultipliers, various light-scattering and fluorescent properties of individual algal cells can be measured simultaneously. For instance, the intensity of the forward blue scatter signal is proportional to the cross-sectional area of a cell [22,31]. Red fluorescence, as measured by the flow cytometer, is linearly related to the chlorophyll concentration of plant cells [32].

A 1-ml aliquot was removed from each flask after 4- and 28-h UV-A exposure for flow cytometric analyses by an Ortho Instruments (Westwood, MA) Cytoflurograf® model 50-H. Measurements were made within 1 h of sample removal. The algal cells were excited by the 488-nm light from an argon laser tuned to 400 mW. Forward blue scatter and red (>620 nm) and green (515–530 nm) fluorescence were measured simultaneously for both unstained cells and cells stained with fluorescein diacetate (FDA). Individual measurements on 5,000 cells were recorded for each sample at flow rates of 200 to 600 cells per second.

Results for the effects of anthracene/UV-A exposure on cell size forward blue scatter and cellular chlorophyll concentrations (red fluorescence) are not reported in this paper because the dose–response relationships for these two flow cytometric measurements were weak or absent. Cell size was significantly greater, relative to the control treatments, in most anthracene/UV-A combinations after 28-h UV-A exposure. However, the dose–response relationship was too weak to perform probit analysis. Increased cellular chlorophyll concentrations were observed in some anthracene/UV-A combinations after 4-h UV-A exposure. However, this was not deemed biologically significant because no apparent dose–response relationship was observed, and after 28-h UV-A exposure, cellular chlorophyll concentrations had returned to control levels in all UV-A/anthracene combinations.

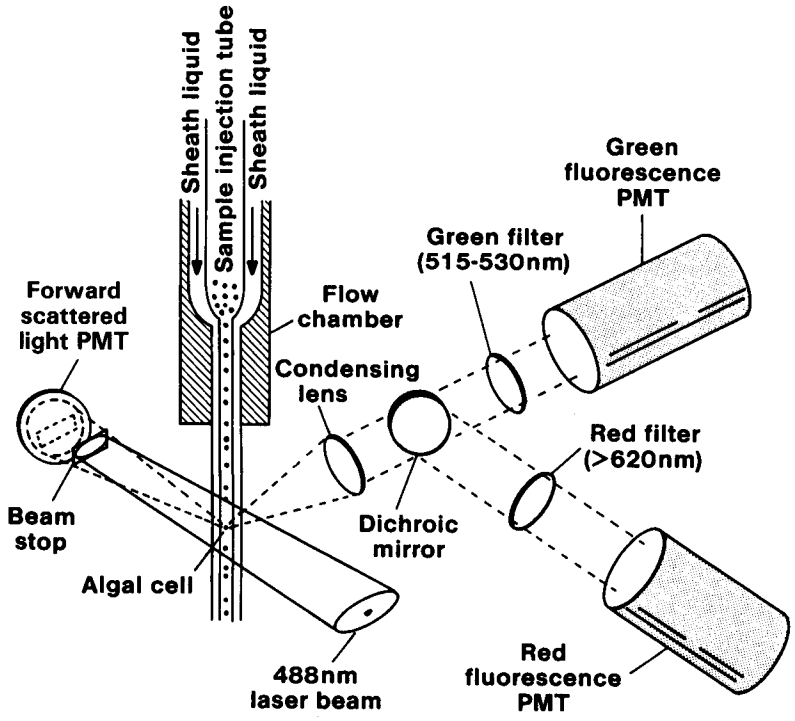

Fig. 3. Schematic representation of the optical configuration of the Ortho Instruments Cytoflurograf® 50-H.

The FDA fluorescence assay is a standard technique [21,23,24,33,34] for determining the viability of a cell. FDA is a lipophilic, nonfluorescent molecule that is readily taken up by cells. Nonspecific esterases within viable cells cleave the ester bonds, producing fluorescein, which when excited with 488-nm light from an argon laser in the flow cytometer fluoresces green [21]. Fluorescein, which is hydrophilic, does not easily leak back out through intact cell membranes. The intensity of the green fluorescence depends primarily on the esterase activity of the cell and, somewhat, on the integrity of the cell membrane [21], both of which are related to cell viability. FDA fluorescence was considered a measure of the physiological "health" of the algal cells.

Cell viability was determined flow cytometrically by staining a 1-ml sample of cells with 10 μl FDA (Sigma F-7378, 1 mg/ml in acetone) for 4 min at 24°C. Fluorescein (green) fluorescence was measured through a 515- to 530-nm bandpass filter. A bimodal distribution of green fluorescence was observed in samples stained with FDA [24]. One group of cells that contained minimal green fluorescence represents cells that did not convert the FDA to the green fluorescent compound fluorescein. These cells contained no esterase activity and were considered nonviable cells. The small amount of green fluorescence observed in these nonviable cells was from background autofluorescence of the photosynthetic pigments. The second group contained cells of varying esterase activity and were considered viable cells. The region designated to contain viable cells started at the upper border of the nonviable region and included the main portion of stained cells. The only excluded cells were cells that were off scale and cells demonstrating abnormally intense green fluorescence (possibly due to recording of doublet cells). Generally, 90% of the stained cells were included in the viable region.

Two measures of cell viability were calculated from the flow cytometry data for each sample. The percentage of unstained cells in the nonviable region (percentage of nonviable cells) was calculated. The nonviable region was delimited in the green vs. red fluorescence cytogram by a rectangle that included >98% of cells in an unstained control sample. In addition, for each sample, a stress index (SI) [21] was calculated. The SI was based on the fact that FDA is cleaved by esterases in the cells to form the fluorescent compound fluorescein. Inhibition of these enzymes or the energy-producing processes in the cells results in a lower rate of cleavage that results in less green fluorescence. The SI was calculated by multiplying the mean FDA (green) fluorescence in the viable region by the number of stained cells in that region. The SI for each sample was corrected for background green fluorescence (SI of unstained control), which was measured before staining. For both measures, the three replicate values were combined to obtain a mean response for each treatment after 4- and 28-h exposures.

Controls

Appropriate controls were performed to ensure that any toxic effects observed were due to the photoinduced toxicity of anthracene. Direct toxicity of anthracene was determined by exposing algal cells in the absence of UV-A radiation to the greatest tested anthracene exposure concentration. Direct UV-A radiation toxicity was investigated by

exposing cells to the maximum UV-A intensity, in the absence of anthracene. The direct and photoinduced toxicity of the primary aqueous photolysis product of anthracene, anthraquinone (Fluka, Ronkonkoma, NY; >99% purity) [28], was also investigated. The toxicity of anthraquinone was tested with a toxicant-renewal system similar to that of the anthracene experiments. At each toxicant renewal, 3.5 μg anthraquinone (equivalent to a target concentration of 35 μg/L) was added. Thus, the algal cells were exposed to anthraquinone concentrations in the anthraquinone control experiment that were greater than the maximum possible anthraquinone concentration if all of the anthracene added in the greatest anthracene exposure was fully converted to anthraquinone.

The toxicity of acetonitrile and any effects of acetonitrile on the photoinduced toxicity of anthracene were determined. The NOEC of acetonitrile, in the presence of UV-A radiation, was determined for all of the flow cytometry end points used in the photoinduced toxicity of anthracene experiments. In all experiments, acetonitrile at exposure conditions identical to those of the photoinduced toxicity experiments had no effect on any flow cytometric end point.

Statistical analysis

An ANOVA was performed for the effect of anthracene on each indicator of toxicity for each UV-A intensity to determine if a significant response was observed. Multiple-range tests were performed to determine if the mean responses of the different anthracene treatments were significantly different from the control treatment (Dunnett's test) or from each other (Tukey's test). The EC50 and EC10, the anthracene exposure concentrations required to reduce the response to 50 and 10%, respectively, of the control treatment at a specific UV-A intensity, were calculated using probit analysis. The flow cytometric end point, percentage of nonviable cells, was not converted to percentage of control, but, rather, the EC values represented the anthracene concentrations required to cause 50% (or 10%) of the cells to be nonviable. All statistical analyses were performed with SAS® [35], except the C.I.s for the EC values were calculated using equations of Neter et al. [36]. Effect isopleths were used to relate effect levels (EC10 and EC50) to combinations of concentrations of anthracene and intensities of UV-A. These isopleths delineated regions that were either sufficient or insufficient to cause the specified degree of effect.

RESULTS

Controls

The effects of UV-A radiation, anthracene, anthraquinone, and the carrier solvent acetonitrile on *S. capricornutum* were determined individually. There was no effect of acetonitrile on any of the measured parameters in the presence or absence of UV-A radiation. A UV-A radiation intensity of 720 μW/cm^2 or less, in the absence of a photosensitizer, caused no observable adverse effects on *S. capricornutum*. Anthracene was directly toxic to algae in the absence of UV-A radiation at an anthracene concentration less than aqueous solubility (35 μg/L). Anthracene in the absence of UV-A radiation was neither lethal (no increase in the percentage of nonviable cells) nor were effects on growth observed [11]. However, after 28-h exposure, flow cytometric measures, cellular chlorophyll and the SI, detected sublethal toxicity at 19.4 μg/L anthracene. Cellular chlorophyll was 18% greater and the stress index was 17% less, relative to cells not exposed to anthracene. Thus, anthracene in the absence of UV-A radiation did cause sublethal alterations in certain cell characteristics, such as cellular chlorophyll concentration and SI. Anthraquinone, the major stable photoproduct of anthracene, was not toxic to *S. capricornutum* in the presence or absence of UV-A radiation (720 μW/cm^2). No effect on any flow cytometric end point was observed in anthraquinone-treated cells compared to the appropriate control. Thus, the observed effects of the combination of anthracene and UV-A radiation were not due to the direct or photoinduced toxicity of anthraquinone in the medium.

Photoinduced toxicity of anthracene

Stress index. Reduction of cell viability, as measured by the SI, due to exposure to anthracene and UV-A radiation was readily apparent after as little as 4 h of UV-A exposure (Fig. 4). *Selenastrum capricornutum* cells exposed to concentrations of anthracene >16 μg/L for 4 h had a significantly lower SI relative to control treatments at all tested UV-A intensities (Fig. 4). The SI was also significantly lower, relative to control treatments, after 4-h UV-A exposure for cells exposed to >5 μg/L anthracene at UV-A intensities >125 μW/cm^2. A dose–response relationship between cell viability, as measured by the SI, and anthracene/UV-A exposure was discernible after 4-h UV-A exposure (Fig. 4). The sensitivity and predictability of the SI demonstrate the utility of the flow cytometric FDA stress index in algal toxicity testing.

Effect thresholds for combinations of anthracene and UV-A radiation were estimated for the SI after 4 or 28 h of UV-A exposure by probit analysis (Table 1, Fig. 5). The determined thresholds were for the minimum anthracene concentration at the maximum UV-A intensity and minimum UV-A intensity at anthracene solubility (35 μg/L) to cause 50 or 10% effect. The effects thresholds were determined by the best nonlinear fit to the resultant probit values. After 4 h, the threshold for a 50% reduction in the SI (EC50) was approximately 12 μg/L anthracene and 100 μW/cm^2 UV-A. The threshold for a 10% reduction in the SI was >3 μg/L anthracene and <50 μW/cm^2 UV-A (Fig. 5).

A significant reduction of the SI after 28-h UV-A exposure (Fig. 4) occurred only at the same combinations of anthracene and UV-A that caused significant inhibition of algal growth rate [11]. A definite threshold was evident for photoinduced toxicity of anthracene to the SI after 28-h UV-A exposure. The 28-h EC50 values for the SI ranged from 3.6 to 16.1 μg/L anthracene, depending on the UV-A intensity (Table 1, Fig. 5). The 28-h EC10 for the SI ranged from 1.6 to 8.3 μg/L anthracene. Direct toxicity of anthracene may have occurred at the least intense UV-A exposure because there did not appear to be a threshold of UV-A radiation for inhibition of cell viability, as measured by the SI. The threshold concentrations of anthracene for reducing the SI after 28-h UV-A exposure by 50 and 10% were approximately 3 and 1 μg/L, respectively.

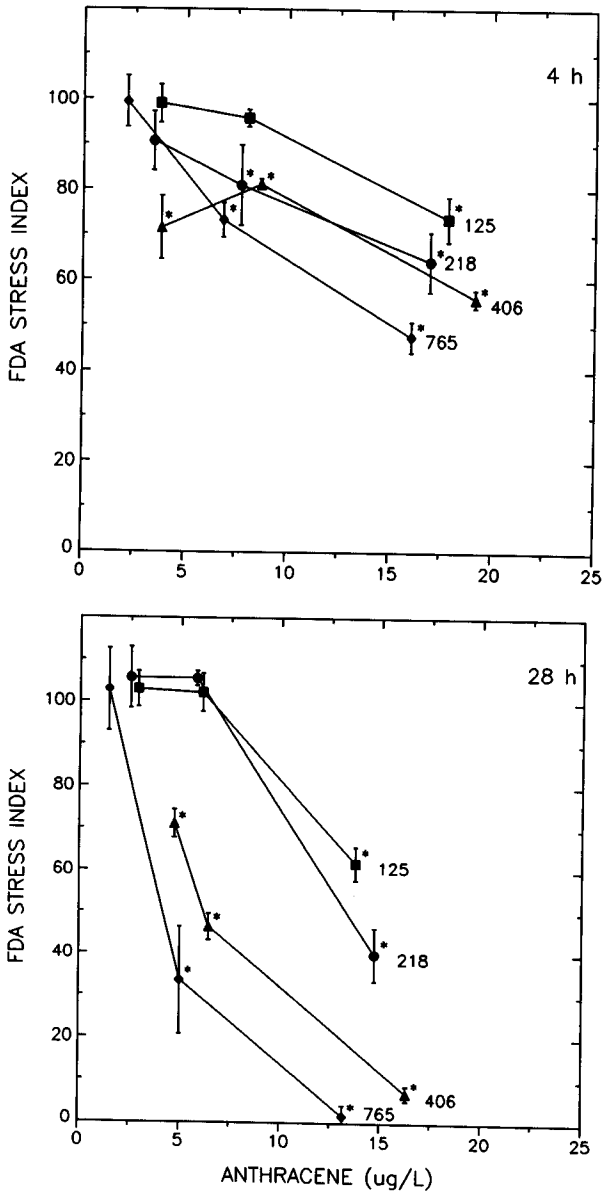

Fig. 4. FDA stress index (SI) for algal cells exposed to anthracene and UV-A radiation. Mean SI was normalized to the control mean (set equal to 100). UV-A intensities in $\mu W/cm^2$ are indicated to the right of each dose–response curve. Error bars represent one standard deviation of the mean. Concentrations with asterisks are significantly different from the control mean (control = 0 $\mu g/L$ anthracene; Dunnett's test, $p < 0.05$).

Nonviable cells. Anthracene or UV-A radiation had minimal effects on percentage of nonviable cells after 4-h UV-A exposure, but the effect was greater after 28 h. All combinations of anthracene and UV-A that inhibited algal growth rate [11] also caused a greater percentage of nonviable cells after 28 h, except for the smallest anthracene treatment at 406 $\mu W/cm^2$ UV-A (Fig. 6). The significant increase in the percentage of nonviable cells observed at 6 $\mu g/L$ anthracene and 125 $\mu W/cm^2$ UV-A (Fig. 6) was not considered toxicologically important because the observed percentage of nonviable cells was within the typical range in the controls (5.8–14.6%), and a significant decrease in the percentage of nonviable cells occurred at the greatest anthracene treatment at 125 $\mu W/cm^2$ UV-A. The proportion of cells determined to be nonviable was not affected by concentrations of anthracene <5 $\mu g/L$ (Fig. 6). The proportion of nonviable cells was directly proportional to the concentration of anthracene and UV-A intensity at greater concentrations.

Thresholds were evident for the effects of both anthracene and UV-A exposure after 28-h exposure (Fig. 7). The threshold for 50% nonviable cells was 5 $\mu g/L$ anthracene and 150 $\mu W/cm^2$ UV-A, and for 10% nonviable cells 2 $\mu g/L$ anthracene and 100 $\mu W/cm^2$ UV-A. The 28-h EC50 and EC10 for cell viability ranged from 4.5 to 15.8 and 1.7 to 6.8 $\mu g/L$ anthracene, respectively (Table 2, Fig. 7).

DISCUSSION

Concentrations of anthracene, less than aqueous solubility, that caused significant effects on the FDA SI had no direct toxic effect on [^{14}C]bicarbonate incorporation in *S. capricornutum* [37]. The EC50 for [^{14}C]bicarbonate incorporation by *Chlamydomonas angulosa* and *Chlorella vulgaris* has been reported to be 245 and 550 $\mu g/L$ anthracene, respectively [38], which is five- to 10-fold greater than the aqueous solubility of anthracene. The detection of the direct toxicity of anthracene in this study at a concentration of 19 $\mu g/L$ anthracene resulted from the use of sensitive flow cytometric measures. However, in previous studies, anthracene, in the absence of UV-A radiation and at a mean exposure concentration of 19.4 μg anthracene per liter, did not have any effect on *S. capricornutum* growth rate [11]. Direct anthracene toxicity caused only an 18% reduction of the SI and a small increase in cellular chlorophyll concentration. Therefore, it appears that direct anthracene toxicity is not important in assessing the environmental hazard posed to algae by anthracene and PAH contamination. However, the detection of direct anthracene toxicity to algae by flow cytometric measures does demonstrate the great sensitivity of flow cytometric techniques.

The use of the green vs. red fluorescence cytogram to delimit nonviable and viable regions is an improvement of the previous method [24], which used only the green fluorescence histogram. By using the green vs. red cytogram, the regions could be set to exclude particles without red fluorescence, which are obviously nonalgal particles. Also, occasionally, especially for greatly inhibited samples, there was no clear delineation between unstained and stained cells when using the green fluorescence histogram. This occurred following FDA staining when small cells did not increase their green fluorescence sufficiently to surpass the background green fluorescence of larger cells. This phenomenon did not pose a problem when using the green vs. red cytogram to delimit nonviable and viable regions.

The SI after 4-h UV-A exposure was a good predictor of inhibition of specific growth rate (0–22 h) caused by exposure to combinations of anthracene and UV-A radiation [11].

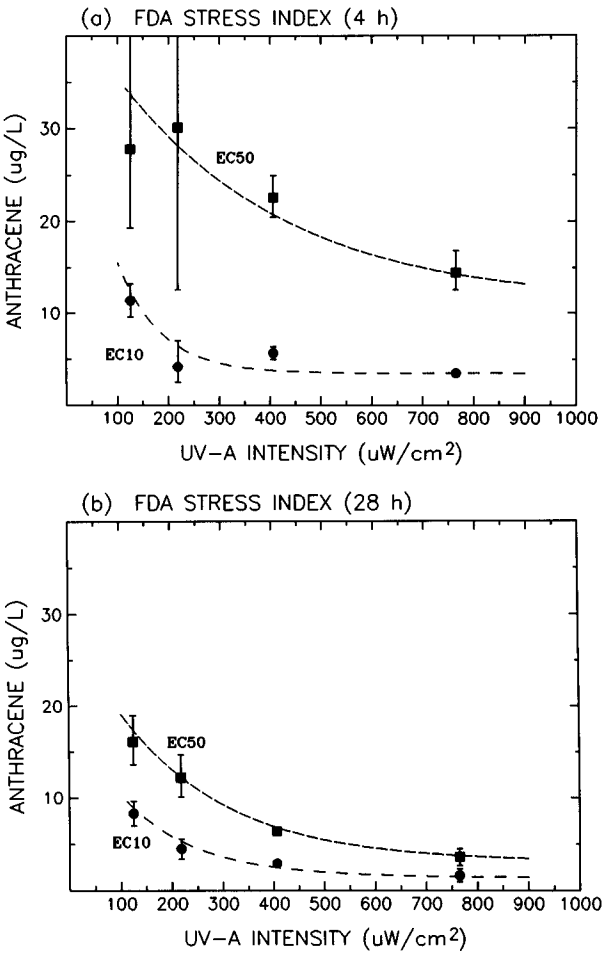

Fig. 5. Effect isopleths for the FDA stress index (SI) of *Selenastrum capricornutum* after 4- and 28-h UV-A exposures. Error bars represent 95% C.I.s.

The EC10 isopleth for the SI after 4-h UV-A exposure (Fig. 5) was identical to the 22-h EC50 isopleth for specific growth rate at UV-A intensities >400 $\mu W/cm^2$ and to the 22-h EC10 isopleth for specific growth rate at smaller UV-A intensities [11]. Again, this indicates that short-term incubation, coupled with flow cytometric measurements, was a sensitive and accurate predictor of the effects of longer term, ecologically relevant responses such as growth rate. In addition, the SI was able to detect sublethal effects of the phototoxicity of anthracene and UV-A, which did not affect the growth rate of the cells.

Algal toxicity end points demonstrated differential sensitivity to the effects of the photoinduced toxicity of anthracene. Growth rate and flow cytometric measures of cell viability were the most sensitive end points to the photoinduced toxicity of anthracene. Photosynthetic end points such as cellular chlorophyll concentration and [^{14}C]bicarbonate incorporation on a per-cell basis were the least sensitive end points [this study; 11]. The 28-h EC50 for the SI was identical to the 22-h EC50 for specific growth rate at all UV-A intensities >200 $\mu W/cm^2$ [11]. At 125 $\mu W/cm^2$ UV-A, the SI was more sensitive than growth rate. Similar sensitivity and EC50 were also observed between the SI and growth rate in sodium dodecyl sulfate- (SDS-) exposed algal cells [24].

The suite of responses of different toxicity end points can be considered characteristic of a toxicant [39]. Analysis of responses of different end points can also elucidate possible mechanisms of toxic action of a toxicant. The photoinduced toxicity of anthracene to algae is characterized by acute algicidal action with inhibition of enzyme systems and cell division occurring before inhibition of photosynthesis. Algicidal toxicants characteristically cause increases in the percentage of nonviable cells in conjunction with inhibition of growth rate [24]. This was the response observed for the photoinduced toxicity of anthracene to *S. capricornutum*. These results suggest that pigments such as carotenoids or other

Table 1. EC50 and EC10 for FDA stress index (SI) of *Selenastrum capricornutum* after 4- and 28-h UV-A exposure

UV-A intensity ($\mu W/cm^2$)	EC50 ($\mu g/L$)	EC10 ($\mu g/L$)	Probit regression equation[a]		
			Slope	y Intercept	r^2
4-h UV-A exposure					
765	14.5 (12.6–16.9)	3.5 (3.0–4.1)	−2.079	7.417	0.98
406	22.6 (20.5–25.0)	5.7 (5.0–6.5)	−2.143	7.902	0.99
218	30.1 (12.6–72.0)	4.2 (2.5–7.0)	−1.504	7.224	0.71
125	27.8 (19.3–40.1)	11.4 (9.7–13.3)	−3.292	9.754	0.93
28-h UV-A exposure					
765	3.6 (2.8–4.7)	1.6 (1.0–2.5)	−3.577	7.008	0.96
406	6.4 (6.1–6.8)	2.9 (2.6–3.3)	−3.716	8.004	0.99
218	12.2 (10.1–14.7)	4.5 (3.5–5.7)	−2.949	8.204	0.98
125	16.1 (13.6–19.0)	8.3 (7.1–9.7)	−4.477	10.401	0.99

ECX is the anthracene concentration required to decrease the SI by X%.
Values in parentheses are 95% C.I.s.
[a]Probit regression equation ($y = a + bx$), where y = probit (% control) and $x = \log_{10}$[anthracene (in $\mu g/L$)].

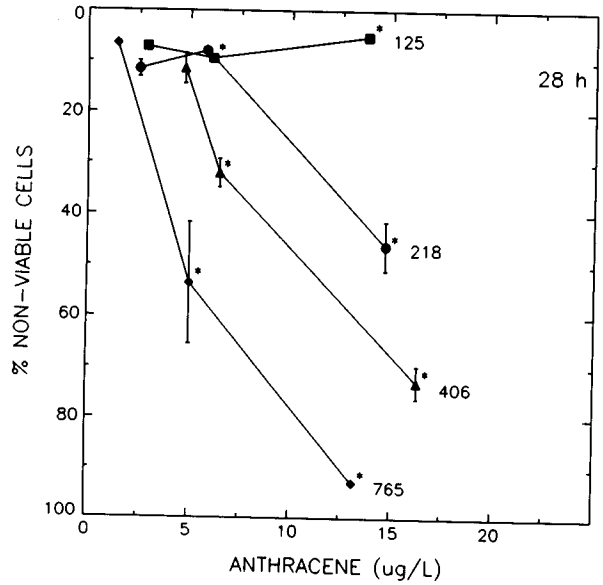

Fig. 6. Cell viability (percentage of nonviable cells) for algal cells exposed to anthracene and UV-A radiation for 28 h. UV-A intensities in $\mu W/cm^2$ are indicated to the right of each dose-response curve. Error bars represent one standard deviation of the mean. Concentrations with asterisks are significantly different from the control mean (control = 0 $\mu g/L$ anthracene; Dunnett's test, $p < 0.05$).

antioxidants may be protecting the chloroplasts [12]. Beta-carotene is an efficient singlet oxygen quencher and is the major photoprotection pigment in algae as well as higher plants [40]. This hypothesis was further tested and corroborated by investigating the effects of fluridone, a herbicide that inhibits the synthesis of carotenoid pigments, on the photoinduced toxicity of anthracene [12]. In those studies, it was found that algal cells, which were deficient in colored carotenoids, were less tolerant of the photoinduced toxicity of anthracene relative to cells that contained the normal complement of colored carotenoids.

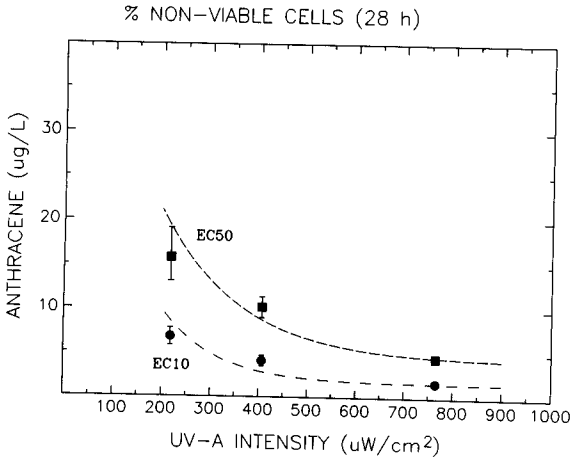

Fig. 7. Effect isopleths for the percentage of nonviable cells of *Selenastrum capricornutum* populations after 28-h UV-A exposure. Error bars represent 95% C.I.s.

Table 2. EC50 and EC10 for cell viability (percentage of nonviable cells) of *Selenastrum capricornutum* after 28-h UV-A exposure

UV-A intensity ($\mu W/cm^2$)	EC50 ($\mu g/L$)	EC10 ($\mu g/L$)	Probit regression equation[a]		
			Slope	y Intercept	r^2
765	4.5 (4.0–5.0)	1.7 (1.5–2.0)	3.075	2.994	0.98
406	10.2 (9.1–11.5)	4.1 (3.5–4.8)	3.230	1.740	0.96
218	15.8 (13.1–19.1)	6.8 (5.8–7.9)	3.474	0.837	0.96
125	Not calculated[b]				

ECX is the anthracene concentration that results in X% nonviable cells.
Values in parentheses are 95% C.I.s.
[a]Probit regression equation ($y = a + bx$), where y = probit (% nonviable) and $x = \log_{10}$[anthracene (in $\mu g/L$)].
[b]Probit regression not calculated because the slope was not significantly different from zero.

Algae were found to be less sensitive to phototoxic effects of anthracene relative to other aquatic organisms. The 28-h EC50 and EC10 for the FDA stress index, the most sensitive algal end point, for *S. capricornutum* were 16.1 and 8.3 $\mu g/L$ anthracene, respectively, at 125 $\mu W/cm^2$ UV-A. Fishes have been found to be slightly more sensitive to anthracene photoinduced lethality. The acute 96-h LC50 and chronic NOEC for juvenile bluegill sunfish exposed continuously to 100 $\mu W/cm^2$ of UV-A radiation have been reported to be 4.5 and 1.2 $\mu g/L$ anthracene, respectively [14]. The 21-d chronic EC50 and EC10 for *Daphnia magna* reproduction at 117 $\mu W/cm^2$ UV-A (16:8-h light:dark photoperiod) were approximately 4.2 and 1.0 $\mu g/L$ anthracene, respectively [17]. The relative sensitivity of aquatic organisms to photoinduced toxicity of anthracene is fish, zooplankton > phytoplankton. However, based on the more sensitive end points measured in this study, algae are not as tolerant to the phototoxic effects as was previously thought. The greater sensitivity of aquatic animals to photoinduced toxic effects, relative to plants, is additional evidence that carotenoids, present in greater concentrations in algae, are providing protection from the photoinduced toxicity of anthracene.

CONCLUSIONS

The flow cytometric methods used in this study proved to be sensitive, predictive measures of the direct and photoinduced toxicity of anthracene and UV-A radiation to *S. capricornutum*. A concentration of 19 $\mu g/L$ anthracene was slightly toxic to *S. capricornutum* in the absence of UV-A radiation. A UV-A intensity of 720 $\mu W/cm^2$, in the absence of photosensitizer, was not toxic to *S. capricornutum*. Anthraquinone was not toxic to *S. capricornutum* in the presence or absence of UV-A radiation. The FDA SI was a sensitive short-term predictor of longer term effects on the population growth rate. The threshold for effects, based on the SI, was 1 $\mu g/L$ anthracene, and there appeared to be no UV-A radiation threshold because of the direct toxic effects of anthra-

cene on the SI. The 28-h EC50 for the SI ranged from 3.6 to 16.1 μg/L anthracene for UV-A intensities between 125 and 750 $\mu W/cm^2$. Measures of the viability of algal cells were more sensitive than measures of photosynthesis. This suggests that carotenoids may be protecting algal cells from the photoinduced toxicity of anthracene.

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